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WITNESS my hand this
Twenty-seventh day of December 1999

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PROVISIONAL SPECIFICATION

Invention Title: "COMBINATORIAL LIBRARIES"

The invention is described in the following statement:

TITLE

"COMBINATORIAL LIBRARIES"

FIELD OF THE INVENTION

THIS INVENTION relates generally to combinatorial compound libraries. In particular, the present invention relates to carriers having unique codes for use in combinatorial compound synthesis as well as to combinatorial compound libraries produced with those carriers. The invention is also concerned with a novel method for structural deconvolution of a combinatorial library member.

BACKGROUND OF THE INVENTION

Recently, there has been substantial interest in devising facile combinatorial technologies to synthesize molecular libraries of immense diversity. A major utility of such libraries is that they can be screened for various biological, pharmacological or chemical activities in the pursuit of novel lead compounds.

In essence, combinatorial technologies are predicated on systematic assembly of a collection of chemical building blocks or synthons in many combinations using chemical, biological or biosynthetic procedures. The potential number "*N*" of

different individual library members produced by such an assembly can be calculated as a function of the number of different synthons available for each step "*b*" and the number of synthetic steps in the reaction scheme "*x*", according to the following formula: $N = b^x$. Thus, a library of nonapeptides constructed using 20 different amino acids (*i.e.*, the synthons) could include as many as 20^9 or 5.1×10^{11} different library members.

Combinatorial libraries may be assembled by a number of methods including the "split-process-recombine" or "split synthesis" method described first by Furka *et al.* (1988, *14th Int. Congr. Biochem.*, Prague, Czechoslovakia **5**:47; 1991, *Int. J. Pept. Protein Res.* **37**:487-493) and Lam *et al.* (1991, *Nature* **354**:82-84), and reviewed later by Eichler *et al.* (1995, *Medicinal Research Reviews* **15(6)**:481-496) and Balkenhohl *et al.* (1996, *Angew. Chem. Int. Ed. Engl.* **35**:2288-2337). The split synthesis method involves dividing a plurality of solid supports such as polymer beads into *n* equal fractions representative of the number of available synthons for each step of the synthesis (*e.g.*, 20 L-amino acids, 4 different nucleotides *etc*), coupling a single respective synthon to each polymer bead of a corresponding fraction, and then thoroughly mixing the polymer beads of all the

fractions together. This process is repeated for a total of x cycles to produce a stochastic collection of up to N^x different compounds. Thus, by employing syntheses where the coupling involves the addition of synthons such as amino acids, nucleotides, sugars, lipids or heterocyclic compounds, where the synthons may be naturally-occurring, synthetic or combinations thereof, one may create a large number of molecularly diverse compounds.

The molecular libraries so produced can then be screened for the identification of novel ligands that interact with a receptor target of interest. For any given receptor target, the probability of successfully identifying a potent ligand through a process of randomly screening molecular repertoires will undoubtedly increase as the size and structural diversity of the library is also increased. However, an inherent difficulty of producing large libraries of this type is the ability to determine the reaction history of any chosen combinatorial library member to thereby deconvolute its structure. For large numbers of solid supports and large numbers of steps and/or processing methods, this "deconvolution" procedure is particularly difficult. In many practical cases, where high throughput and fast analysis is required, this problem is intractable by conventional methods.

The conventional split synthesis technologies referred to above present difficulties when it is desired to detect and isolate a combinatorial library member of interest. In this regard, it is necessary to first cleave the member from its solid support before identifying the member by techniques such as mass spectroscopy or HPLC. This is time consuming and cumbersome and in some cases, cleavage is not possible.

A number of groups have attempted to overcome these prior art deficiencies by use of chemical encoding which relies on reactions different and orthogonal from those used in the synthesis of the combinatorial library member. For example, Janda (1994, *Proc. Natl. Acad. Sci. USA* **91**:10779-10785) describes a method in which each synthesis step of a combinatorial library member is followed by an independent coupling of an identifier tag to a solid support. Through a series of sequential chemical steps, a sequence of identifier tags are built up in parallel with the compounds being synthesized on the solid support. When the combinatorial synthesis is complete, the sequence of operations any particular solid support has gone through may be retraced by separately analyzing the tag sequence. Accordingly, use of identifier tags in this manner provides a means

whereby one can identify which synthon reaction an individual solid support has experienced in the synthesis of a combinatorial library member. The identifier tag also records the step in the synthesis series in which the solid support visited that synthon reaction. In this regard, reference may be made to International Publication WO93/06121 in which Dower *et al.* disclose a general stochastic method for synthesizing a combinatorial compound library on solid supports from which library members may be cleaved to provide a soluble library. The identifier tag may be attached directly to a member of the library with or without an accompanying particle, to a linker attached to the member, to the solid support on which the member is synthesized or to a second particle attached to the member carrying particle.

However, while Dower *et al.* (*supra*) refer very broadly to the types of identifier tags that may be utilized in combinatorial library formation, the only experimental evidence that they provide is the use of oligonucleotides as tags which are identifiable by sequencing or hybridization. They also make reference to amplifying the oligonucleotide tag by PCR if only trace amounts of oligonucleotide are available for analysis. However, it will be appreciated that such identification methods are time consuming and

inefficient. For example, use of PCR may result in PCR product contamination making it necessary to introduce further measures to overcome this problem as described by Dower *et al.* (*supra*). It is also necessary to sequence amplified DNA and this involves an additional step in the identification procedure.

In U.S. Patent No. 5,721,099, Still *et al.* disclose a process of constructing complex combinatorial chemical libraries of compounds wherein each compound is produced by a single reaction series and is bound to an individual solid support on which is bound a combination of four distinguishable identifiers which differ from one another. The combination provides a specific formula comprising a tag component capable of analysis and a linking component capable of being selectively cleaved to release the tag component. Each identifier or combination thereof encodes information at a particular stage in the reaction series for the compound bound to the solid support. The identifiers are used in combination with one another to form a binary or higher order encoding system permitting a relatively small number of identifiers to be used to encode a relatively large number of reaction products. However, the method of Still *et al.* (*supra*) does not provide for direct identification of the tag component

on the solid support. In this regard, it is essential prior to analysis of a combinatorial library that each tag component be cleaved from the support thus creating at least one additional step which is time consuming and inefficient. Accordingly, the same disadvantages relevant to the method of Dower *et al.* also apply to that of Still *et al.*

In addition to the disadvantages mentioned above, chemical encoding techniques such as those described by Junda (1994, *supra*), Dower *et al.* (*supra*) and Still *et al.* (*supra*) rely on parallel, orthogonal synthesis of identifier tags which adds substantially to the time taken for completion of a combinatorial synthesis and has the potential to interfere with the synthesis.

Spectrometric encoding methods have also been described in which decoding of a library member is permitted by placing a solid support directly into a spectrometer for analysis. This eliminates the need for a chemical cleavage step. For example, Geysen *et al.* (1996, *Chem. Biol.* **3**:679-688) describe a method in which isotopically varied tags are used to encode a reaction history. A mass spectrometer is used to decode the reaction history by measuring the ratiometric signal afforded by the multiply isotopically labeled tags. A disadvantage of this

method is the relatively small number of multiply isotopically labeled reagents that are commercially available.

Optical encoding techniques have also been described in which a solid support's absorption or fluorescence emission spectrum is measured. For example, reference may be made to Sebestyén *et al.* (1993, *Pept. 1992 Proc. 22nd Eur. Pept. Symp.* 63-64), Campian *et al.* (1994, In *Innovation and Perspectives on Solid Phase Synthesis* Epton, R., Birmingham: Mayflower, 469-472) and Egner *et al.* (1997, *Chem. Commun.* 735-736) who describe the use of both chromophoric and/or fluorescent tags for bead labeling in peptide combinatorial synthesis. Although this use provides advantage for deconvoluting a library member's structure by simply reading a bead's absorption or fluorescence emission spectrum, the encoding of a large library would require the use of many chromophores or fluorophores where spectral superimposition would be a likely drawback.

Yamashita and Weinstock (International Publication WO 95/32425) disclose the coupling on beads of (i) fluorescently labeled tags having intensities that differ by a factor of at least 2, and/or (ii) multiple different fluorescent tags that can be used in varying ratios, to encode a

combinatorial library. Such beads may be used in concert with flow cytometry to construct a series of combinatorial libraries by split synthesis procedure. In this regard, a first combinatorial library is prepared by conducting a specified set of reaction sequences on tagged beads according to (i) and (ii) to encode each choice of synthon in the first stage of combinatorial synthesis (the term *"stage"* corresponds to a step of a sequential synthesis of a combinatorial library member). A second combinatorial library is prepared from substantially the same specified set of reaction sequences as the first library wherein the tagged beads are combined and separated prior to the first reaction sequence and the beads are sorted prior to the second reaction sequence to encode each choice of synthon in the second stage. The sorting step is characterized in that the beads are sorted into groups of similarly tagged beads. Additional libraries are prepared according to the preparation of the second library except that the sort step is performed prior to a different stage in the combinatorial synthesis. The number of libraries constructed in the series will therefore equal to the total number of stages in the combinatorial synthesis wherein a different stage is encoded in each library. After synthesis is complete, each library is tested for biological activity and a

population analysis analogous to Structure Activity Relationship (SAR) studies is conducted for each library to reveal which variable synthon(s) are important for activity and which are not. Although this method has advantages in relation to providing a lead structure, it is necessary to construct and analyze multiple libraries commensurate with the number of stages used for the combinatorial synthesis which is cumbersome and time consuming.

Kaye and Tracey (International Publication WO 97/15390) describe a physical encoding system in which chemically inert solid particles are each labeled with a unique machine readable code. The code may be a binary code although higher codes and alphanumerics are contemplated. The code may consist of surface deformations including pits, holes, hollows, grooves or notches or any combination of these. Such deformations are applied by micromachining. Alternatively, the code may reside in the shape of the particle itself. Solid particles comprising a first phase for combinatorial synthesis and a second phase containing a machine readable code are exemplified wherein the second phase may be superimposed on, or encapsulated within, the first phase. The microscopic code on the particles may be interrogated and read using a microscope-based image capture and processing

system. The encoding system of Kaye and Tracey provides advantage in that the machine readable code may be read *"on-the-fly"* between process steps of a combinatorial synthesis thus allowing the process sequence, or audit trail, for each bead to be recorded. However, this system suffers from a number of drawbacks in that specialized purpose-built machinery is required for producing the solid particles and for reading the code. For example, the application of code deformations onto the solid particles requires expensive micromachining technology, computer aided design (CAD) tools for designing the required particle geometry, as well as manufacture of appropriate photolithographic masks for delineating the particle shapes. In addition, there is a need to utilize specialized image processing systems and software for observing a particle from several different directions to accurately determine and verify a given code.

Many of the disadvantages of the known methods described above as well as many of the needs not met by them are addressed by the present invention which, as described more fully hereinafter, provides numerous advantages over the above-described methods.

DISCLOSURE OF THE INVENTION

According to one aspect of the invention, there is provided a carrier having a reaction platform upon which a compound can be synthesized, and having a combination of at least two features integrally associated therewith, wherein said features are detectable during synthesis of a compound, and wherein one or more of said at least two features is a light emanating or light absorbing feature detectable by illuminating the carrier with incident light of one or more selected wavelengths or of one or more selected vectors.

In another aspect, the invention provides a plurality of carriers, wherein each carrier has a reaction platform upon which a compound can be synthesized, and wherein substantially each carrier has a unique code characterized by a combination of at least two features integrally associated with the carrier, and wherein said features are detectable during synthesis of a respective compound, and wherein one or more of said at least two features is a light emanating or light absorbing feature detectable by illuminating a respective carrier with incident light of one or more selected wavelengths or of one or more selected vectors.

By "*features integrally associated with the carrier*" is meant features of the carrier and/or features of one or more elements, molecules, groups, tags and the like associated with the carrier.

Any light emanating feature may be employed, For example, the light emanating feature may be selected from the group consisting of light scattering, luminescence, phosphorescence and atomic or molecular fluorescence emission. It will be appreciated that techniques including, but not limited to, 2 photon and 3 photon time resolved fluorescence spectroscopy as for example disclosed by Lakowicz *et al.* (1997, *Biophys. J.*, **72**:567, incorporated herein by reference); fluorescence lifetime imaging as for example disclosed by Eriksson *et al.* (1993, *Biophys. J.*, **2**:64, incorporated herein by reference); pump probe microscopy as for example disclosed by Dong *et al.* (1997, *Optik*, **106**:7, incorporated herein by reference); Raman spectroscopy as for example disclosed by Rahman *et al.* (1998, *J. Org. Chem.*, **63**:6196, herein incorporated by reference) may be used in this regard.

Suitably, the light scattering may result from diffraction, reflection, polarization or refraction of the incident light. In this regard, the carriers may be formed of different materials to

provide a set of carriers with varying scattering properties such as refractive indexes.

The fluorescence emission may result from excitation of one or more fluorescent tags attached to the carrier. In the case of two or more fluorescent tags being utilized, the tags may be the same wherein the tags contain varying amounts of a fluorophore and are therefore intensity-differentiated. Alternatively, the tags may be different wherein they are present in a ratio of 1:1 or varying ratios. Reference may be made in this regard to Yamashita *et al.* (International Publication WO 95/32425) which is herein incorporated by reference.

Exemplary fluorophores which may be used in accordance with the present invention include those discussed by Dower *et al.* (International Publication WO 93/06121 which is incorporated by reference herein). Preferably, fluorescent dyes are employed. Any suitable fluorescent dye may be used for incorporation into the carrier of the invention. For example, reference may be made to U.S. Patents 5,573,909 (Singer *et al.*, which is incorporated herein by reference) and 5,326,692 (Brinkley *et al.*, which is incorporated herein by reference) which describe a plethora of fluorescent dyes. Reference may also be made to fluorescent dyes described in U.S. Patent Nos.

5,227,487, 5,274,113, 5,405,975, 5,433,896, 5,442,045, 5,451,663, 5,453,517, 5,459,276, 5,516,864, 5,648,270 and 5,723,218 which are all incorporated herein by reference.

One or more of the fluorescent dyes are preferably incorporated into a microparticle, such as a polymeric microparticle or ceramic microparticle. Such microparticles may be attached to the carrier by use of colloidal interactions as for example disclosed by Trau and Bryant in copending International Application PCT/AU98/00944 which is incorporated herein by reference.

The polymeric microparticle can be prepared from a variety of polymerizable monomers, including styrenes, acrylates and unsaturated chlorides, esters, acetates, amides and alcohols, including, but not limited to, polystyrene (including high density polystyrene latexes such as brominated polystyrene), polymethylmethacrylate and other polyacrylic acids, polyacrylonitrile, polyacrylamide, polyacrolein, polydimethylsiloxane, polybutadiene, polyisoprene, polyurethane, polyvinylacetate, polyvinylchloride, polyvinylpyridine, polyvinylbenzylchloride, polyvinyltoluene, polyvinylidenechloride and polydivinylbenzene. The microparticles may be prepared from styrene monomers. Ceramic

microparticles may be comprised of silica, alumina, titania or any other suitable transparent material.

A suitable method of making silica microparticles is described, for example in "The Colloid Chemistry of Silica and Silicates" Cornell University Press by Ralph K Iler 1955 which is herein incorporated by reference.

The microparticles may be of any suitable size or shape. For example, the microparticles may be shaped in the form of spheres, cubes, rectangular prisms, pyramids, cones, ovoids, sheets or cylinders. Typically, microparticles which may be used in the present invention have a diameter of about 0.01 μm to about 50 μm.

Fluorescent dyes may be incorporated into microparticles by any suitable method known in the art, such as copolymerization of a polymerizable monomer and a dye-containing comonomer or addition of a suitable dye derivative in a suitable organic solvent to an aqueous suspension as for example disclosed in Singer *et al.*, (*supra* including references cited therein), Campian *et al.* (1994, In *Innovation and Perspectives on Solid Phase Synthesis* Epton, R., Birmingham: Mayflower, 469-472, herein incorporated by reference) and Egner *et al.* (1997, *Chem. Commun.* 735-736, herein incorporated by

reference). Alternatively, fluorescent microparticles may be produced having at least one fluorescent spherical zone. Such particles may be prepared as for example described in U.S. Patent No. 5,786,219 (Zhang *et al.*) which is incorporated herein by reference.

The light absorbing feature may result from absorbance of light by the carrier. Fourier transform infrared spectroscopy as for example described by Rahman *et al.* (1998, *J. Org. Chem.*, **63**:6196, incorprated by reference herein) may be used in this regard.

Of course it will be appreciated that features other than light emanating features or light absorbing features may be used. Such features may include surface deformations of the carrier inclusive of pits, holes, hollows, grooves or notches or any combination thereof. Alternatively, the feature may include chromophoric, radioactive, magnetic, metallic or luminescent labels.

The carriers may comprise any solid material capable of providing a base for combinatorial synthesis. For example, the carriers may be polymeric supports such as polymeric beads which are preferably formed from polystyrene crosslinked with 1-5% divinylbenzene. Polymeric beads may also be formed from hexamethylenediamine-polyacryl resins and related

polymers, poly[N-{2-(4-hydroxylphenyl)ethyl}] acrylamide (i.e. (one Q)), silica, cellulose beads, polystyrene beads poly(halomethylstyrene) beads, poly(halostyrene) beads, poly(acetoxystyrene) beads, latex beads, grafted copolymer beads such as polyethylene glycol/polystyrene, porous silicates for example controlled pore-glass beads, polyacrylamide beads for example poly(acryloylsarcosine methyl ester) beads, dimethylacrylamide beads optionally cross-linked with N,N'-bis-acrylolyl ethylene diamine, glass particles coated with a hydrophobic polymer inclusive of cross-linked polystyrene or a fluorinated ethylene polymer which provides a material having a rigid or semi-rigid surface, poly(N-acryloylpyrrolidine) resins, Wang resins, Pam resins, Merrifield resins, PAP and SPARE polyamide resins, polyethylene functionalized with acrylic acid, kieselguhr/polyamide (Pepsyn K), polyHipe, PS/polydimethylacrylamide copolymers, CPG, PS macrobeads and Tentagel, PEG-PS/DVB copolymers.

These carrier materials will usually contain functionalities or be able to be functionalized for attachment of reporter beads or linkers. Suitable functionalities include $-NH_2$, -COOH, -SOH, -SSH or sulfate groups.

It will also be appreciated that the polymeric beads may be replaced by other suitable supports such as pins or chips as is known in the art, e.g. as discussed in Gordon *et al.* (1994, *J. Med. Chem.* **37(10)**:1385-1401). The beads may also comprise pellets, discs, capillaries, hollow fibers or needles as is known in the art.

Reference is also made to International Publication WO93/06121, which is incorporated herein by reference, which describes a broad range of supports that may constitute carriers for use in present invention. By way of example, these carriers may be formed from appropriate materials inclusive of latex, glass, gold or other colloidal metal particles and the like. Reference may also be made to International Publications WO95/25737 or WO97/15390 which are herein incorporated by reference to examples of suitable carriers.

The carriers may have any suitable size or shape.

It will be appreciated from the foregoing that the number of carriers having different detectable codes will be dependent on the number of different features integrally associated with the carriers. For example, code heterogeneity may be achieved simply by use of carriers of different shapes

and/or sizes, and/or by use of carriers which are formed of different materials. Alternatively, the code heterogeneity may be effected by use of carriers having different tags and/or different combinations of tags integrally associated therewith. By *"tag"* is meant any molecule or groups of molecules having one or more recognizable features including, but not restricted to, shape, size, color, optical density, differential absorbance or emission of light, chemical reactivity, magnetic or electronic encoded information, or any other distinguishable feature.

The term "compound" as used herein comprises a sequence of synthons which includes any structural unit that can be formed and/or assembled by known or conceivable synthetic operations. For example, synthons may include amino acids, carbonates, sulfones, sulfoxides, nucleosides, carbohydrates, ureas, phosphonates, lipids, esters or combinations thereof. Alternatively, the synthons may comprise inorganic units such as for example silicates and aluminosilicates.

Compounds which may be synthesized on the carriers include, but are not limited to, linear, cyclic and branched polymers of nucleic acids, polysaccharides, phospholipids and peptides having either alpha-, beta- or omega- amino acids,

heteropolymers, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, or other polymers, as will be readily apparent to one skilled in the art. The numbers quoted and the types of compounds listed are merely illustrative and are not limiting.

In particular, the carriers of the invention are applicable to any type of chemical reaction that can be carried out on a solid support. Such chemical reaction includes, for example:-

(i) [2 + 2] cycloadditions including trapping of butadiene;

(ii) [2 + 3] cycloadditions including synthesis of isoxazolines, furans and modified peptides;

(iii) acetal formation including immobilization of diols, aldehydes and ketones;

(iv) aldol condensation including derivatization of aldehydes, synthesis of propanediols;

(v) benzoin condensation including derivatization of aldehydes;

(vi) cyclocondensations including benzodiazepines and hydantoins, thiazolidines, -turn mimetics, porphyrins, phthalocyanines;

(vii) Dieckmann cyclization including cyclization of diesters;

(viii) Diels-Alder reaction including derivatization of acrylic acid ;

(ix) electrophilic addition including addition of alcohols to alkenes;

(x) Grignard reaction including derivatization of aldehydes;

(xi) Heck reaction including synthesis of disubstituted alkenes;

(xii) Henry reaction including synthesis of nitrile oxides *in situ* (see [2 + 3]cycloaddition);

(xiii) catalytic hydrogenation including synthesis of pheromones and peptides (hydrogenation of alkenes);

(xiv) Michael reaction including synthesis of sulfanyl ketones, bicyclo]2.2.2]octanes;

(xv) Mitsunobu reaction including synthesis of aryl ethers, peptidyl phosphonates and thioethers;

(xvi) nucleophilic aromatic substitutions including synthesis of quinolones;

(xvii) oxidation including synthesis of aldehydes and ketones;

(xviii) Pausen-Khand cycloaddition including cyclization of norbornadiene with pentynol;

(xix) photochemical cyclization including synthesis of helicenes;

(xx) reactions with organo-metallic compounds including derivatization of aldehydes and acyl chlorides;

(xxi) reduction with complex hydrides and Sn compounds including reduction of carbonyl, carboxylic acids, esters and nitro groups;

(xxii) Soai reaction including reduction of carboxyl groups;

(xxiii) Stille reactions including synthesis of biphenyl derivatives;

(xxiv) Stork reaction including synthesis of substituted cyclohexanones;

(xxv) reductive amination including synthesis of quinolones;

(xxvi) Suzuki reaction including synthesis of phenylacetic acid derivatives; and

(xxvii) Wittig, Wittig-Horner reaction including reactions of aldehydes; pheromones and sulfanyl ketones.

Reference may also be made to Patel *et al.*, April 1996, DDT **1(4)** 134-144 which refers to manufacture or synthesis of N-substituted glycines, polycarbamates, mercaptoacylprolines, diketopiperazines, HIV protease inhibitors, 1-3 diols, hydroxystilbenes, B-lactams, 1,4-benzodiazepine-2-5-diones, dihydropyridines and dihydropyrimidines.

Reference may also be made to synthesis of polyketides as discussed in Rohr, 1995, Angew. Int. Ed. Engl. **34** 881-884.

Linkers for use with the carriers may be selected from base stable anchor groups as described in Table 2 of Fruchtel *et al.*, 1996, *supra* or acid stable anchor groups as described in Table 3 of Fruchtel *et al.*, 1996, *supra*. In this regard, the Fruchtel *et al.*, 1996, reference is incorporated herein by reference. Linkers for use with the carriers of the invention are also referred to in International Publication WO93/06121 which is herein incorporated by reference.

Generally the anchors developed for peptide chemistry are stable to either bases or weak acids but for the most part, they are suitable only for the

immobilization of carboxylic acids. However, for the reversible attachment of special functional groups, known anchors have to be derivatized and optimized or, when necessary, completely new anchors must be developed. For example, an anchor group for immobilization of alcohols is (6 hydroxymethyl)-3,4 dihydro-2H-pyran, whereby the sodium salt is covalently bonded to chloromethylated Merrifield resin by a nucleophilic substitution reaction. The alcohol is coupled to the support by electrophilic addition in the presence of pyridinium toluene-4 sulphonate (PPTS) in dichloromethane. The resulting tetrahydropyranyl ether is stable to base but can be cleaved by transetherification with 95% trifluoroacetic acid.

Benzyl halides may be coupled to a photolabile -sulphanyl-substituted phenyl ketone anchor.

It will also be appreciated that compounds prepared with the carriers and/or process of the present invention may be screened for an activity of interest by methods well known in the art. For example, such screening may be effected by flow cytometry as for example described by Needels *et al.* (1993, *Proc. Natl. Acad. Sci. USA* **90**:10700-10704, herein incorporated by reference), Dower *et al.* (*supra*), and Kaye and Tracey (International

Application WO 97/15390, incorporated by reference herein).

Compounds that may be so screened include agonists and antagonists for cell membrane receptors, toxins, venoms, viral epitopes, hormones, sugars, cofactors, peptides, enzyme substrates drugs inclusive of opiates and steroids, proteins including antibodies, monoclonal antibodies, antisera reactive with specific antigenic determinants, nucleic acids, lectins, polysaccharides, cellular membranes and organibles.

In yet another aspect, the invention resides in a method of producing a plurality of substantially uniquely encoded carriers, comprising the steps of:

(i) preparing a plurality of carriers having different codes wherein each code is characterized by a combination of at least two detectable features;

(ii) detecting the at least two features of each carrier to thereby assign a code for each carrier;

(iii) identifying carriers having distinctive codes;

(iv) identifying carriers having similar codes; and

(v) separating the carriers having distinctive codes from the carriers having non-distinctive codes to thereby provide a plurality of carriers having detectably unique codes.

Suitably, the step of detecting is further characterized in that at least three, preferably at least four, more preferably at least five and most preferably at least six different features of a respective carrier are detected for code recordal. The inventors have found in this regard that the more features one can detect, the greater the number of carriers that will have a detectably unique code.

The identification steps (steps (iii) and (iv)) may be effected by use of any suitable method or apparatus for analyzing the detectable features of a carrier. Preferably, these steps are effected by flow cytometry. For example, a flow cytometer may be used to determine forward scatter (which is a measure of size of a carrier), side scatter (which is a measure of refractive index of a particle), and fluorescent emission.

Suitably, the step of separating is effected by flow cytometry.

In a further aspect, the invention provides a method of synthesizing and deconvoluting a combinatorial library comprising the steps of:-

(a) suspending a plurality of carriers in a fluid, wherein each carrier has a reaction platform upon which a compound can be synthesized, and wherein substantially each carrier has a unique code characterized by a combination of at least two features detectable during synthesis of a respective compound, and wherein one or more of said at least two features is a light emanating or light absorbing feature detectable by illuminating a respective carrier with incident light of one or more selected wavelengths or of one or more selected vectors;

(b) dividing the fluid containing the carriers into a plurality of portions;

(c) determining and recording the code by illuminating the carriers with incident light of one or more selected wavelengths or of one or more selected vectors, wherein said codes are determined during or after the division

step (step (b)) in order to track the movement of specific carriers into said respective portions;

(d) subjecting respective portions to specific chemical reactions;

(e) recombining the respect portions;

(f) iterating steps (b), (c), (d) and (e) as necessary to create a combinatorial compound library in which substantially each member of the library is associated with one or more carriers with a code, wherein tracking data is available to identify the sequence of reactions experienced by substantially each carrier.

Preferably, the codes are determined by flow cytometry.

The invention in yet a further aspect refers to a combinatorial compound library comprising a plurality of different compounds having a multiplicity of different synthons which library has been formed by the aforementioned method.

The invention in a still further aspect resides in a kit comprising:-

a combinatorial compound library including a plurality of different compounds wherein each compound

is attached to a respective carrier, and wherein substantially each carrier has a unique code characterized by a combination of at least two features detectable during synthesis of a respective compound, and wherein one or more of said at least two features is a light emanating or light absorbing feature detectable by illuminating a respective carrier with incident light of one or more selected wavelengths or of one or more selected vectors; and

tracking data on each code to identify the sequence of reactions experienced by each carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Reference will now be made to a preferred embodiment of the invention with reference to the attached drawings, in which:-

FIG. 1 is a schematic representation of one step in a split-process-recombine procedure, *e.g.* as discussed in the prior art in relation to the synthesis of peptide libraries;

FIG. 2 is a schematic representation of the entire iterative split-process-recombine procedure referred to in FIG. 1;

FIG. 3 is a schematic representation of an example of a multi-parameter flow cytometer having multiple lasers set up in series, and accompanying

detectors which can measure a plurality of different fluorescence wavelengths and light scattering at various angles;

FIG. 4A is a graph of the side scatter (SSC-H) and forward scatter (FSC-H) profiles of a plurality of microspheres; and

FIG. 4B is a graph of the side scatter (SSC-H) and forward scatter (FSC-H) profiles relating to a sorted fraction of the plurality of microspheres shown in FIG. 4A.

DESCRIPTION OF PREFERRED EMBODIMENT

Flow cytometry for the determination of combinatorial chemistry reaction histories

A split-process-recombine procedure involving m steps, say step 1, step 2, … , step m, and n(i) processes at step i (i=1,2,…,m) may be defined as follows. For i=1,2,…,m, let the n(i) processes at step i be $P_1(i), P_2(i), \ldots, P_{n(i)}(i)$. At each step i=1,2,...,m:

- the carriers are partitioned, at random but possibly in specific ratios, into n(i) subsets $S_1(i), S_2(i), \ldots, S_{n(i)}(i)$;
- for j=1,2,...,n(i) process $P_j(i)$ is performed on the carriers in subset $S_j(i)$;
- the carriers are recombined.

A schematic representation of this procedure is shown in FIGS. 1 and 2.

Examples of such processes include the combinatorial synthesis of oligonucleotide and oligopeptide chains. In these examples, insoluble polymer beads (colloidal particles, typically 1-1000 μm in diameter) may be used as the carriers onto which nucleic or amino acid monomers are attached and sequentially grown. By performing a split-process-recombine procedure repeatedly for a large number of carriers, a large variety of randomly generated oligonucleotide or polypeptide sequences can be synthesised. Each carrier thus contains an attached polymer with a unique sequence which is defined by the sequence of processing events which the carrier has experienced (*i.e.*, the specific path which the carrier has followed in FIG. 2).

The present invention relates to a novel and convenient method to determine the sequence of processes applied to each of the carriers involved in a split-process-recombine procedure. This procedure involves, for $i=1,2,\ldots,m$ and $j=1,2,\ldots,n(i)$, passing the carriers in the subset $S_j(i)$ through a flow cytometer to obtain a signature or code for each of the carriers present in the subset. The code of each carrier will be determined by a combination of features of the

carriers as described above. The coding data is stored for the purpose of determining the sequence of processes (*i.e.*, reaction history of the carrier) applied to each of the carriers.

The code of a particular carrier for which the process history is required is checked against the list of codes which has been stored for each subset $S_j(i)$. The set of subsets $S_j(i)$ in which the particular carrier's signature occurs determines the set of processes $P_j(i)$ which have been performed on the carrier and hence its entire process history.

It is important therefore that the code of any carrier be reproducible and distinguishable from the code of any other carrier which is used in the split-process-recombine procedure. Reproducibility may be enhanced through, for example, the use of silica carriers. Split-process-recombine procedures may be employed in the manufacture of carriers in order to facilitate efficient production of extremely large numbers of distinguishable particles. In a preferred embodiment, flow cytometry is used to sort and remove subpopulations of indistinguishable carriers. However, partial or complete determination of process histories which are sought may be obtained without perfect code distinction and reproducibility. For example, if two particles become detectably

indistinguishable in the seventh step of a 10-step split synthesis, then the reaction history of either particle through steps 8 to 10 may be used to deduce the reaction history those particles.

In addition, modern flow cytometers have multiple capabilities, such as four-way sorting, multi-laser beam excitation and an upgradable format for attachment of extra filters and detectors (FIG. 3). Current commercial flow cytometers can advantageously perform up to twelve simultaneous measurements separately or in multiparametric fashion. They incorporate multi-laser beam excitation with simultaneous measurement of parameters such as fluorescence intensity, light scattering at various angles, Coulter volume and time. Time-of-flight measurements can be also performed. In multiparametric analysis, the data from several detectors is stored as matrices preserving the association of events. These flow cytometers can also physically separate (sort) subpopulations of particles on the basis of any parameter or combination of parameters. Leading edge technology in the field provides extremely high throughput screening and sorting with rates of up to 100,000 particles per second.

EXAMPLE

Sample preparation involved mixing 10.2 μm polystyrene/divinylbenzene microspheres (Duke Scientific Corp., Cat. No. 7520A, CV = 14.7%, 10 μl) with 21.7 μm polystyrene/divinylbenzene microspheres (Duke Scientific Corp., Cat. No. 7520A, CV = 14.7%, 10 μl) and diluting with 5 ml Milli-Q water. The sample was sonicated for 30 minutes.

The sample was passed through a FACSCalibur flow cytometer (Becton Dickinson) and the side scatter and forward scatter of 7575 events were recorded (FIG. 4A). Each event corresponds to a dot in FIG. 4A but not all events are particles: there is noise in the system. Three hundred particles with side scatter values between 260 and 356 and forward scatter values between 140 and 180 were collected and passed through the cytometer a second time. This region is known as the gated region and events within the gated region can be separated from the remainder of the sample using the sorting capability of the flow cytometer. The results of the second pass are shown in Figure 4B. The event dense region of FIG. 4B superimposes on the gated region of FIG. 4A proving that variation of side scatter and forward scatter for individual particles is minimal. The dense region of FIG. 4B is within the

side scatter values of 252 and 365 and within forward scatter values of 133 and 185.

Thus, it will be appreciated that microspheres can be sorted, by use of forward scatter and side scatter parameters, into a first population having indistinguishable light scattering properties and into a second population in which substantially each microsphere has a unique distinguishable light scattering property. Those of skill in the art will also appreciate that the number of microspheres in the second population can be increased simply by employing greater than two parameters and preferably three or more parameters as for example described herein.

Dated this Thirtieth day of November, 1998

THE UNIVERSITY OF QUEENSLAND,

by their Patent Attorneys,

FISHER ADAMS KELLY.

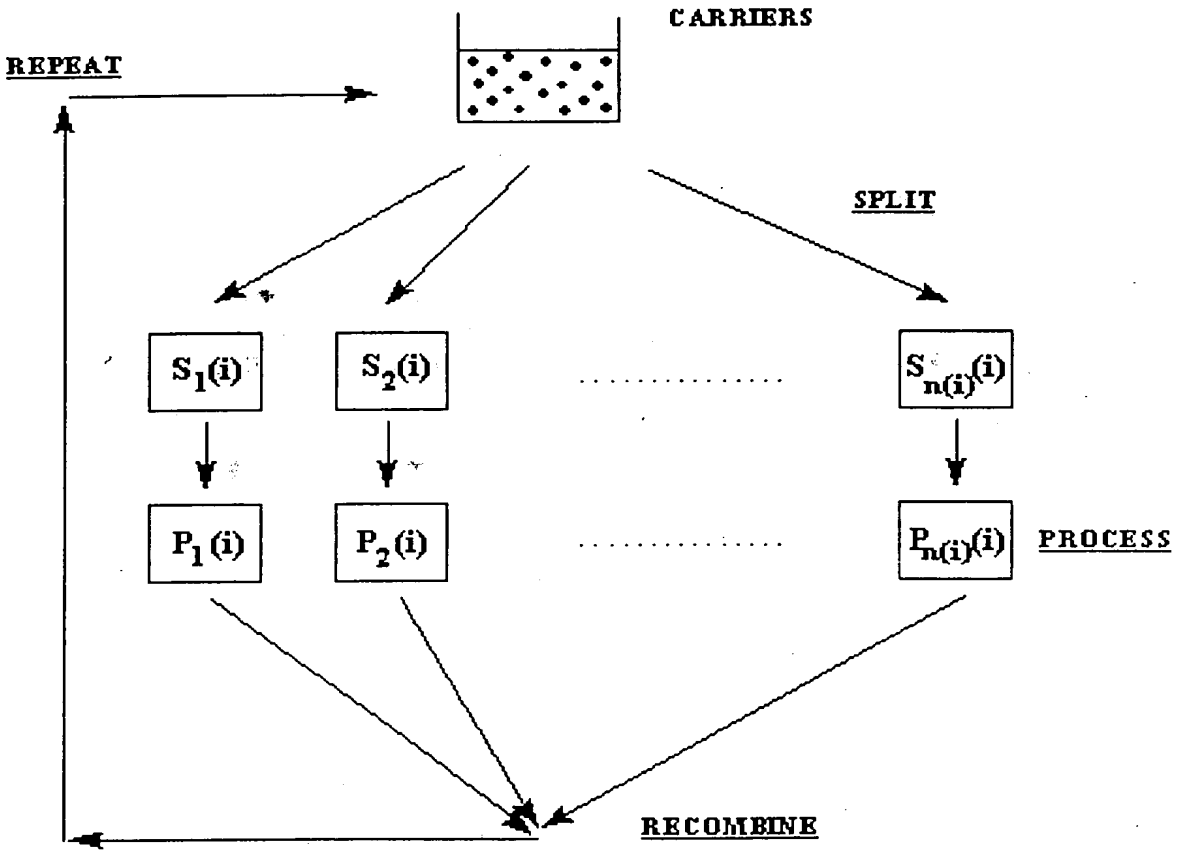
CARRIERS
REPEAT
SPLIT
$S_1(i)$
$S_2(i)$
$S_{n(i)}(i)$
$P_1(i)$
$P_2(i)$
$P_{n(i)}(i)$
PROCESS
RECOMBINE

FIG. 1

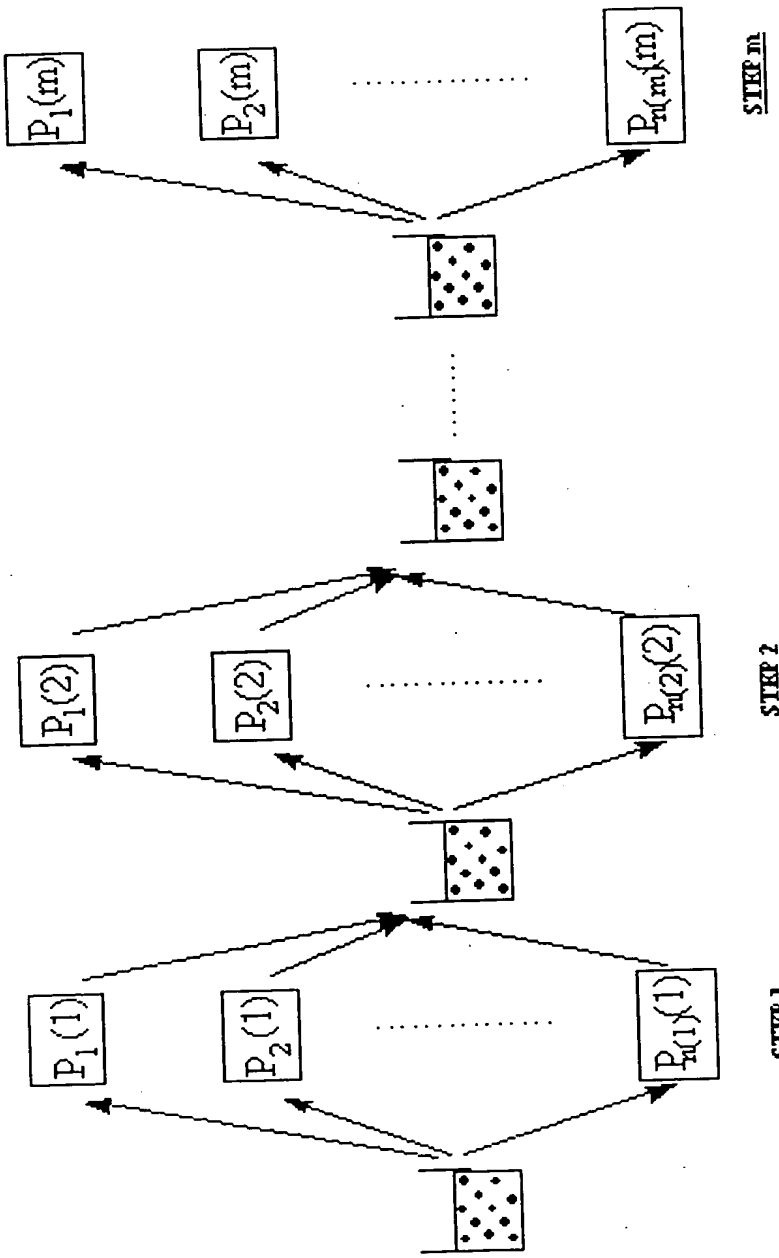

FIG. 2

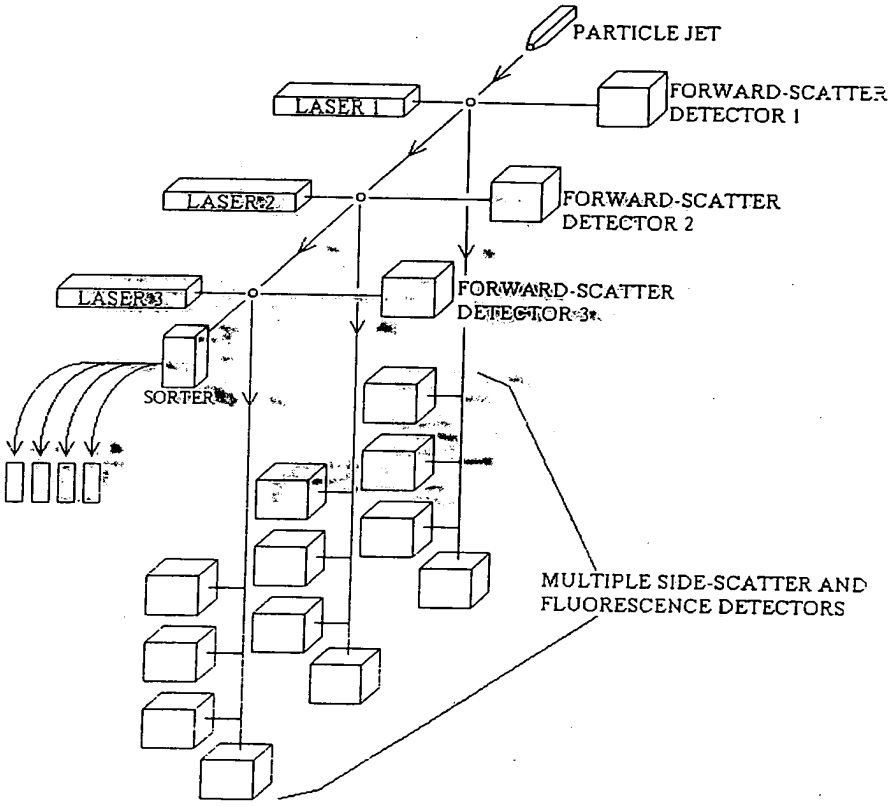
PARTICLE JET
LASER 1
FORWARD-SCATTER DETECTOR 1
LASER 2
FORWARD-SCATTER DETECTOR 2
LASER 3
FORWARD-SCATTER DETECTOR 3
SORTER
MULTIPLE SIDE-SCATTER AND FLUORESCENCE DETECTORS

FIG. 3

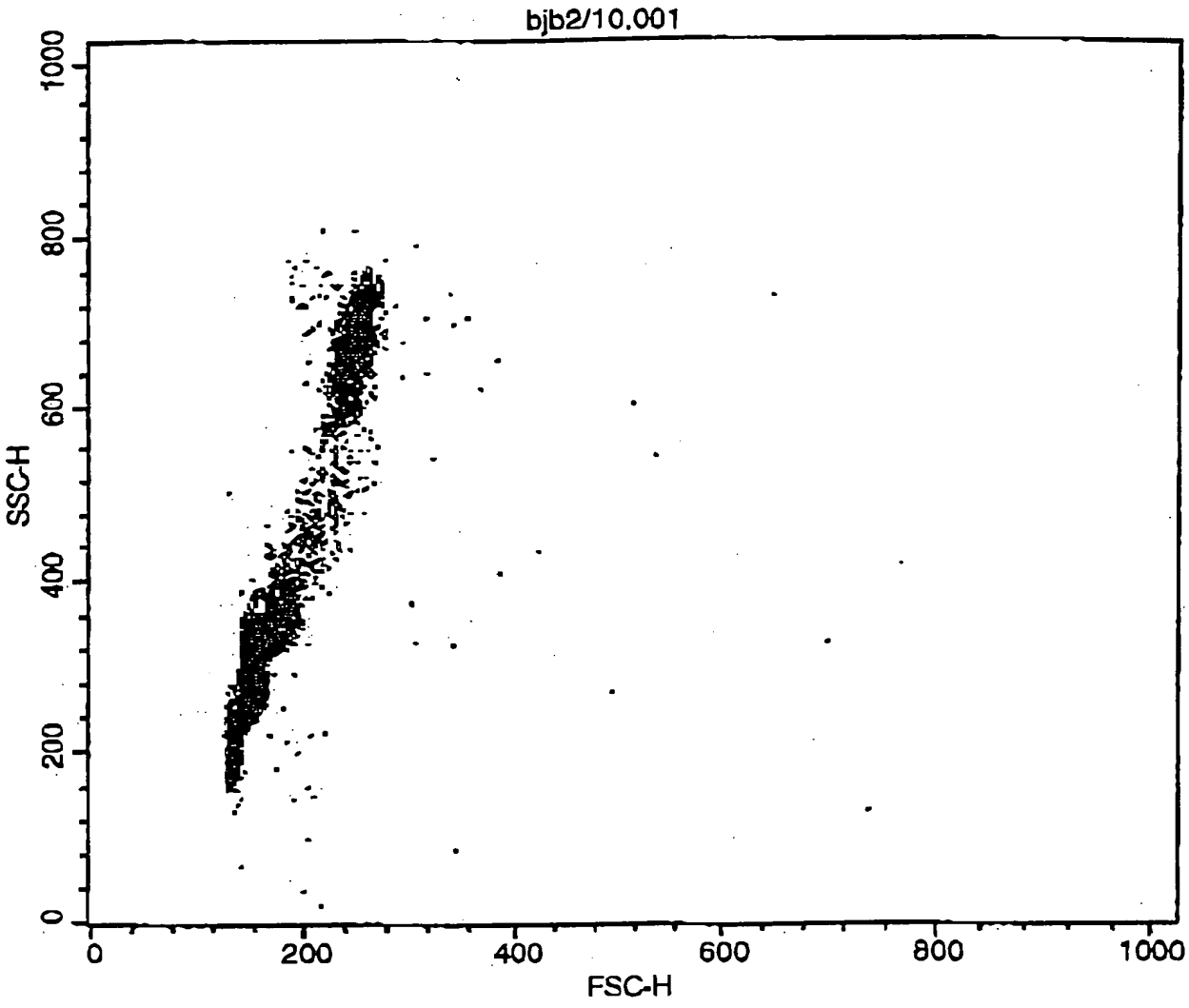

FIG. 4A

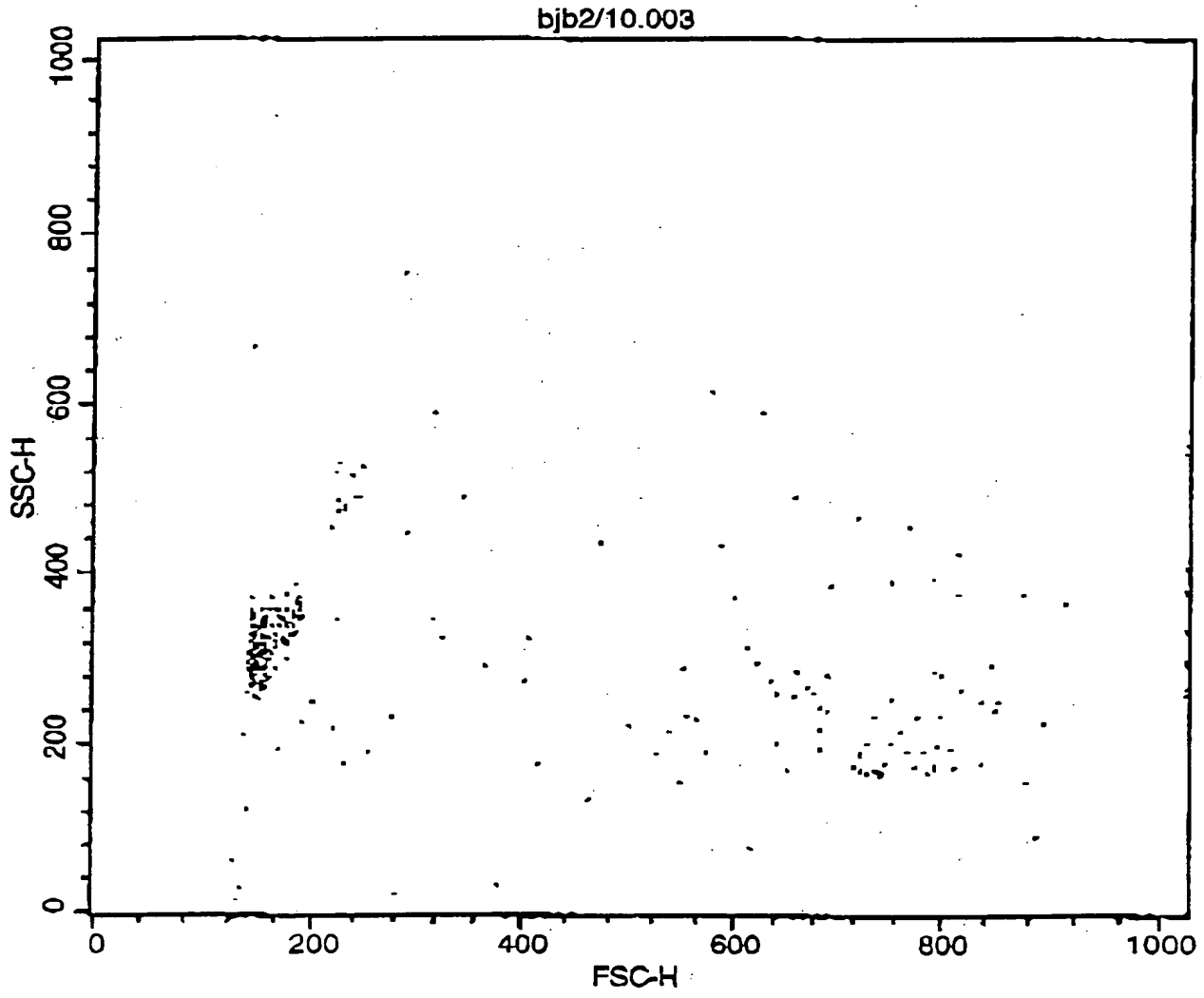

FIG. 4B